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Molecular Detection of 16srrna of *Mycoplasma Pneumoniae, Chlamydia Pneumoniae* and *Legionella Pneumophila* in Community-Acquired Pneumonia

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Abstract: Approximately one third of community- acquired pneumonia cases are caused by atypical pneumonia agents. These agents are *Mycoplasma pneumoniae, Chlamydia pneumoniae* and *Legionella pneumoniae.* The laboratory diagnosis of these organisms is difficult and time-consuming by conventional microbiological techniques. Polymerase chain reaction (PCR) is one of the important tools which can resolve this problem. Among 45 bronchoalveolar lavage specimens taken from patients presented clinically with community-acquired pneumonia. PCR results that 10 cases (22.2%) gave positive for *Mycoplasma pneumoniae* , 8 cases (17.7%) gave positive for *Chlamydia pneumoniae* and 3 (6.6%) cases gave positive results for *Legionella pneumonphila* . The PCR method is a rapid, sensitive and specific technique that has been applied to the detection of many infectious pathogens.

Keywords: Community-acquired pneumonia, PCR, Atypical Pathogens.

INTRODUCTION

Community-acquired pneumonia (CAP) is commonly defined as an acute infection of the lower respiratory tract occurring in patients who not resided in a hospital. Current approaches to the empirical management of CAP emphasise the type of patient (community or hospital) , rather than the type of symptoms (typical or atypical) [1,2] . Distinguishing pneumonia from upper respiratory infections,

particulary bronchitis, is difficult based on symptoms alone. Sustained high fever, chills and pleuritic chest pain suggest pneumonia, but these are not always present [3].

CAP affects approximately 4-5 million adults in the United States annually. About one third of these adults require hospitalization. The mortality rate among hospitalized patients with CAP varies each year and reach 35 percent. Atypical pathogens are responsible for 30-40 of cases and may be copathogens in other cases [4].

Atypical pathogens including *Chlamydia pneumonia, Mycoplasma pneumonia* and *Legionella pneumophila* are an important cause of CAP. *Mycoplasma pneumonia* is primarily a respiratory pathogen that is responsible for approximately 15-20 % of all CAP [5,6] .

Current methods for the identification of atypical pathogens include culturing, rapid antigen detection assays, serology and molecular techniques. Cell cultures for detection of *C. pneumonia* require specialized laboratory and are expensive, time-consuming and labor-intensive. Since *M. pneumoniae* and *L. pneumophila* grow slowly and lack sensitivity, the clinical usefulness of cultures is limited [7]. Because of the non-specificity in clinical presentation of atypical pneumonia, specialized laboratory tests are necessary to establish the diagnosis. The PCR method is a rapid, sensitive and specific technique that has been applied to the detection of many infections pathogens. Different PCR-based assays for the detection of *M. pneumonia, C. pneumonia* and *L. pneumophila* in clinical specimens have been described [4,6] .

Few reports were done in this category for CAP patients in Iraq, so this study is aimed to diagnosis of atypical pathogens in bronchoalveolar lavage (BAL) samples.

MATERIALS AND METHODS

Fourty five patients from both sexes and different age groups were included in this study from some hospitals in Basrah city. The 45 bronchoalveolar lavage (BAL) specimens were kindly provided from those patients, these specimens were taken according to researcher [8] .The patients were examined by physician on admission with signs and symptoms of atypical community acquired pneumonia.

From each case BAL sample was taken: each sample was divided into two portions, one portion was processed for Gram stain, Ziehl-Neelsen stain and culture, the other portion was kept in the frozen till PCR processing for diagnosis of atypical pneumonia.

Nucleic acid extraction:

DNA extraction: Bacterial DNA was extracted from BAL samples using QIAmp DNA Mini Kit with a bacterial DNA extraction protocol. Three ml of sample was centrifuged for 30 min at 20,000 x g. The pellet was resuspended in 180 µl of buffer ATL (QLAGEN Valencia, Calif.) with 20 µl of proteinase K and then incubated at 56 ċ with occasional vortexing until the pellet was completely lysed , which usually took 30 min. After lysis of the sample, 200 µl of buffer AL. was added to the sample and the mixture was incubated for 10 min at 70 ċ. The mixture was then combined with 200 µl of absolute ethanol and mixed by pulse-vortexing for 15 second. The mixture was applied to a spin colum, which holds a silica gel membrane , and spun for 1 min at 6,000 x g. The spin colum was washed with 500 µl of buffer AW2 by centrifugation at 20,000 x g for 3 min. The DNA bound on a membrane was eluted by centrifugation with 50 µl of buffer AE after a5-min incubation at room temperature. The resulting DNA extracts were stored at -20 ċ until PCR assessment.

DNA amplification

Chlamydia pneumoniae: PCR : The extracted DNA were subjected to PCR with primers specific for *C. pneumoniae* omp 1 : 5- TTA TTA ATT GAT GGT ACA ATA- 3 and 5- ATC TAC GGC AGT AGT ATA GTT-3 (PCR base product 207 bp) [9] . In brief , 5 µl of DNA extracts was processed in a 25-µl reaction volume containing PCR buffer (10 Mm Tris [PH 9] , 50 Mm KCL , 0.01% gelatin), 200 µM deoxynucleoside triphosphate , 3.5 Mm MgCL2 , 0.5 µM each primer , and 1 U of Taq polymerase (Promega , Madison , Wis.) . Amplification was carried out in a thermacyler. The first cycle, consisting of a 5-min denaturation at 94 ċ, was followed by 50 cycles each of 30 s at 94 ċ, 45 s at 50 ċ , and 1 min , 30 s , at 72 ċ , with a final extension for 10 min at 72 ċ . The PCR products were visualized in 2% agarose gel containing 0.5 µg of ethidium bromide/ ml.

PCR amplication and electrophoresis:

Legionella pneumophila: To detect *L. pneumophila* the LMP 1-2 primers described by Jaulhac were chosen [10]. The extracted DNA were subjected to PCR with primers specific for *L. pneumophila* . Forward primer (5- AGGGTTGATAGGTTAAGAGC) and reverse primer (5- CCAACAGCTAGTTGACATCG) , PCR base product 451 bp . This pair of primers targets the MIP gene. PCR mixes were prepared in a total volume of 50 µl containing 0.2 mM of each Dntp, 0.3 µM of each primer , 2 Mm mgcl2 , 0.5 U of Taq DNA polymerase , and PCR buffer (10 mM tris HCL PH 8.3 , 50 mM KCL) . After amplification (**Table 1)**, 10 µl of the products were analyzed by agarose gel electrophoresis in (tris-borate –EDTA) TBE buffer , and DNA was stained with ethidium bromide [11] .

Table 1: Amplification conditions used in the protocol of *L.pneumophila DNA*

Amplification condition	*Legionella PCR*
Reaction mixture	
Primer concentration	0.3 µM
MgCL2 concentration	2 mM
Dntp CONCENTRATION	0.2 mM
Taq polymerase	0.5 U
Amplification program	
Denaturation	92 ċ for 1.5 min.
Annealing	62 ċ for 1.5 min.
Extension	72 ċ for 1.5 min.
Cycle number	40

µM: MICROMOLE , mM : milimole , U : Unit

Mycoplasma pneumoniae:

PCR: *M. pneumoniae* –specific PCR was performed with a total reaction mixture volume of 25 µl depending on the researcher [12]. Each primer at a final concentration of 1 µM (primer MP-F (5- CCCT CGACCAAGCCAACCTC-3) and primer MP-R (5- TGCGCGTTGTTCTTGTTGGTG-3) , PCR base product 309 to 339 bp, each deoxynucleoside triphosphate at a final concentration of 200 µM , and final concentration of 10 mM Tris-HCL (PH 8.3) , 50 mM KCL , 2 mM MgCL2 , and 2.5 U of Taq polymerase (Promega , Madison ,Wis.) were used for PCR in a thermocycler (Uno II, Biometra) . Thermocycler conditions consisted of an initial incubation of 95 ċ for 9 min., followed by 40 cycles of 94 ċ for 30 s, 62 ċ for 30 s,and 72 ċ for 30 s. An additional incubation at 72 ċ for 7 min. was added to complete the elongation. The amplified products were visualized in 2% agarose gel containing 0.5 µg of ethidium bromide/ml.

Statistical analysis: The data were analyzed using Chi-square test by SPSS. Differences were considered significant when P< 0.05 [13].

RESULTS

The cases were clinically presented with CAP. Among 45 BAL samples , PCR results showed that 10 (22.2 %) specimens were positive for *Mycoplasma pneumoniae* , 8 (17.7 %) were positive for *Chlamydia pneumoniae* and 3 (6.6 %) were positive for *Legionella pneumophila* (**Figure-1**) . In other hand, there was no statistically significant difference between gender (males and females) and these species of the bacteria. Patients ranged in age from (<1-90 years) . Age group distribution showed that the peak age was between 31-60 years of as age (**Figure-2**). In addition, photograph of agarose gel electrophoresis can be seen in **Figures (3, 4 and 5).**

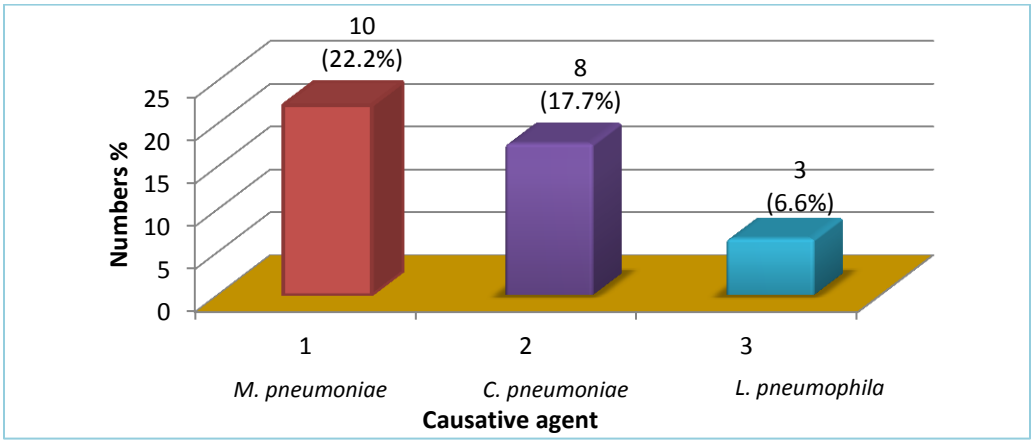

Figure (1): Percentage of PCR assay in 45 BAL specimens

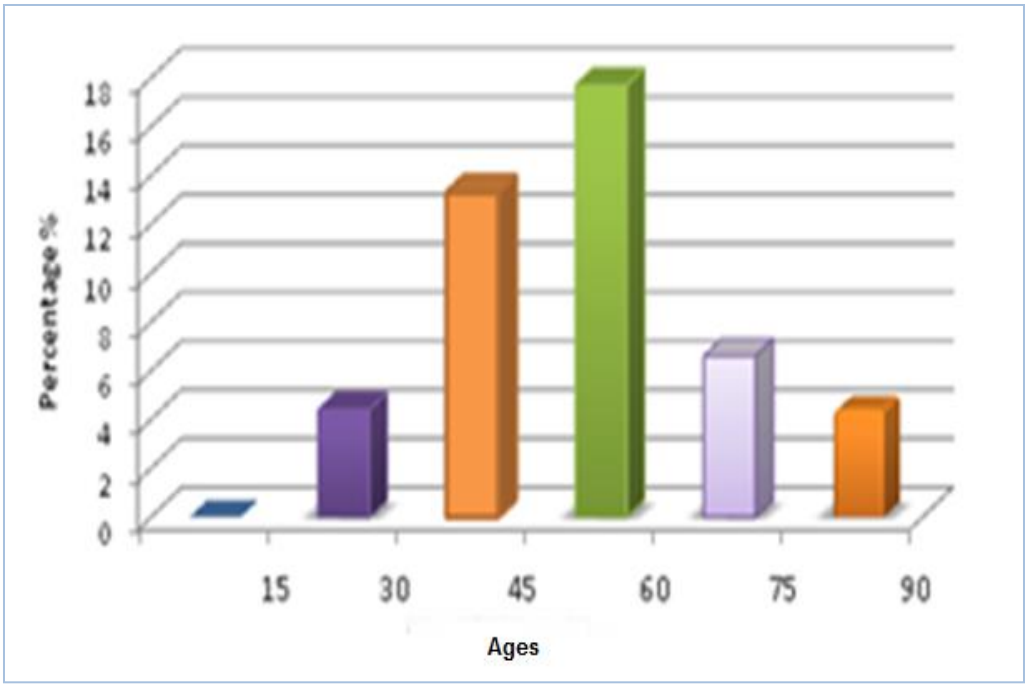

Figure (2): Number of patients infected with community-acquired Pneumonia

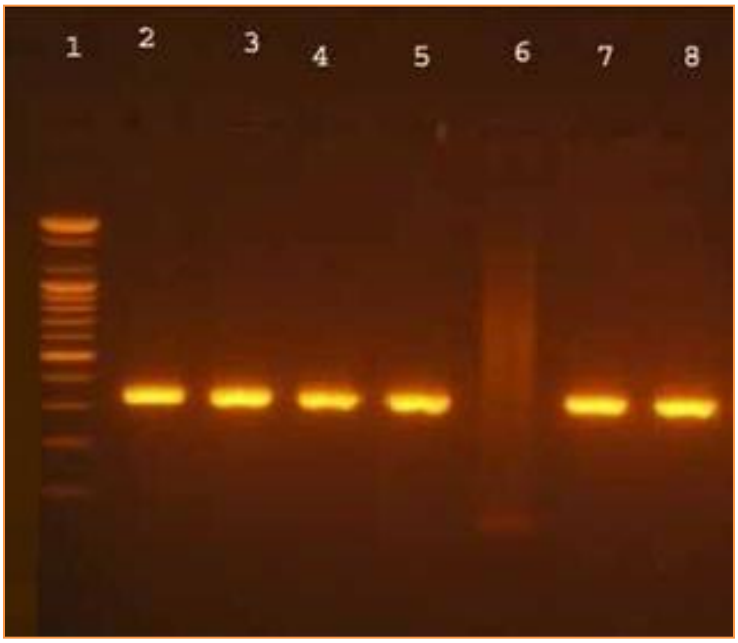

Figure (3): Gel electrophoresis 309-339 bp-positive M.pneumoniae - PCR PCR

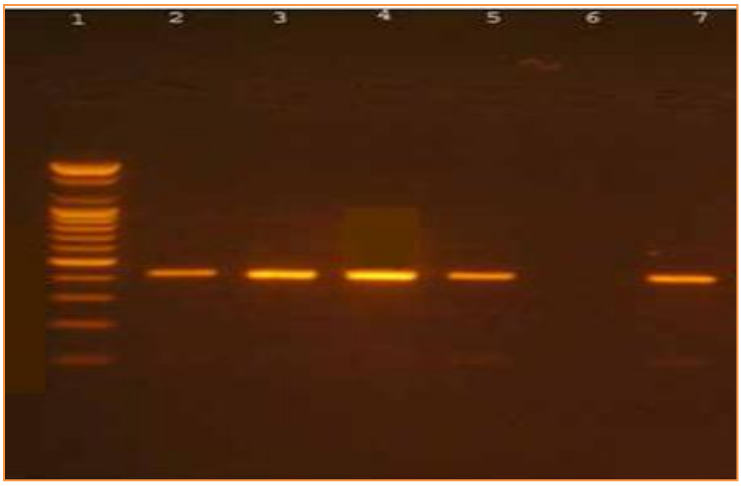

Figure (4): Gel electrophoresis- 451 bp positive *c.pneumoniae* by PCR

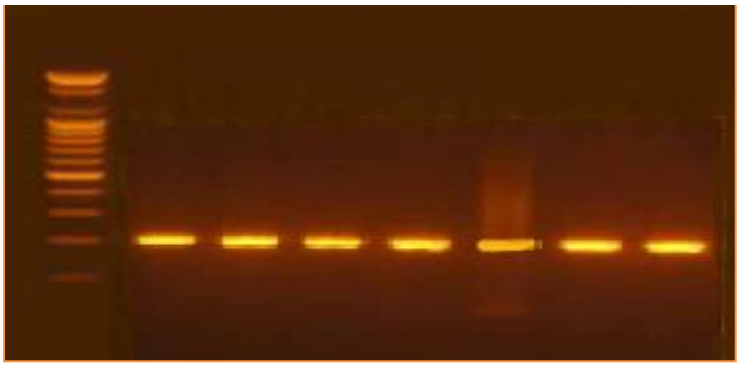

Figure (5): Gel electrophoresis- 207 bp positive *L.pneumoniae* by PCR PCR

DISCUSSION

In other to identify the etiology of respiratory tract infections, clinicians have historically divided the patients clinical and laboratory presentation into (Typical) and (Atypical) pneumonia syndromes. Atypical pneumonia is generally defined as a mild respiratory tract illness with nonproductive cough, fever, headache and abnormal chest X-Ray which progresses from upper to lower respiratory tract [14]. The etiologic diagnosis of infections with atypical pathogens such as *C. pneumoniae* , *M. pneumoniae* and *L. pneumophila* still remains difficult. This is mainly due to difficulties in culturing and to the delayed results associated with conventional methods (serology and culturing). Focus technologies has developed a PCR-based assay for the detection of these pathogens [15, 16].

The results of our study about PCR assay for atypical bacteria of the DNA extract of the BAL specimens reveals that 10 (22.2 %) cases for *M. pneumoniae*, 8 (17.7 %) cases were positive for *C. pneumoniae* and 3 (6.6 %) were positive for *L. pneumophila*. These results are similar with the results of several

previous studies. These studies give variable positivity rates for *M. pneumonia*, ranging from 1% to 27 % [17, 18]. Positive cases for these atypical pathogens more than likely depend on the patient population, socioeconomic factors, age and possibility of exposure.

Pneumonia caused by *M. pneumoniae* has long been a difficult disease to diagnosis because there are both clinical and laboratory diagnostic problems associated with its identification. It has been realized for quite some time that the detection of *M. pneumoniae* is greatly enhanced by the use of the PCR methodology. PCR methods have provided an advantage because they are fast, specific and sensitive[19]. PCR amplification of fragments of the P1 gene or the16 S r RNA gene was shown to be considerably more sensitive than culture for the detection of *M. pneumoniae* [20].

C. pneumoniae is a major cause of acute respiratory disease in human and is responsible for approximately 10% of cases of community-acquired pneumoniae. Due to the difficulties with culturing and serologic analysis, a number of nucleic acid amplification assays for detecting *C. pneumoniae* have been developed. Current PCR methods are based on the amplification of a cloned pst Fragment gene encoding 16S r RNA [21, 22]. While the primer designed to the conserved area of the mip gene for *L. pneumophila* [22].

The age distribution of the 45 patients showed that the peak age was between 31-60 years, as our group of patients may be living in an air conditional closed atmosphere throughout the year.

CONCLUSION

Molecular tests for laboratory diagnosis of infectious agents are getting more importance today. Molecular technique (PCR) was highly sensitive and specific.

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